Gelatinase Expression in Generalized Epidermolysis Bullosa Simplex Fibroblasts

Jan-Olof Winberg, Cand. Real. and Tobias Gedde-Dahl, M.D.
Department of Genetics, The Norwegian Radiumhospital, Oslo, Norway

The use of gelatinase expression in dermal fibroblast cultures as a marker for generalized epidermolysis bullosa simplex (D-EBS-Köbner) has been tested. None of the 6 Köbner patients tested (from 3 families) produced reduced amounts of gelatinase compared with their healthy relatives and other control groups. This shows that a reduced production of gelatinase from dermal fibroblasts is not uniformly a marker for D-EBS-K. *J Invest Dermatol 87: 326–329, 1986*

The hereditary and acquired forms of the disease epidermolysis bullosa (EB) can be separated into at least 16 clinical and genetic types, with the dominant EB simplex as the most common form [1]. Dominant EB simplex (D-EBS) is electron microscopically characterized by cytolysis of the subnuclear cytoplasm in the basal epidermal cells [2,3]. In spite of its epidermal localization, studies of dermal fibroblasts have revealed unexpected results. In a Finnish family with generalized D-EBS-Köbner (D-EBS-K), a decrease in galactosylhydroxylysyl glucosyltransferase production in dermal skin fibroblasts was reported to be associated with the disease [4]. In another study dermal skin fibroblasts from 3 patients with D-EBS-K and 6 of 13 patients with the localized D-EBS Weber-Cockayne (D-EBS-WC) produced extremely small amounts of gelatinase [5]. This trait was suggested to be a marker for the D-EBS-K form of the disease. If confirmed, this would have important implications for the development of a prenatal diagnosis test for severely affected families.

The purpose of the present study was to test fibroblasts from EB families previously characterized by Gedde-Dahl and Anton-Lamprecht [6,7] to determine whether a decrease in gelatinase is: (1) a uniform marker for D-EBS-K: (2) associated to D-EBS-K in some families because of genetic heterogeneity or because of close genetic linkage between the D-EBS gene locus on the one hand and a structural or regulatory gelatinase gene locus on the other hand; or (3) an accidental event.

MATERIALS AND METHODS

Materials Bovine serum albumin (BSA), acid-soluble calf skin collagen, NAD, NADH, 4-methylumbelliferylacetate, 4-methylumbelliferone, *d,l*-lactate, Coomassie Brilliant Blue G-250, trypsin, and soybean trypsin inhibitor (SBTI) were from Sigma. Culture media and fetal calf serum were from Flow Laboratories and GIBCO. Ready Solv HP was from Beckman and tritiated sodium borohydride was from Amersham.

Subjects Skin fibroblast cultures were established from normal volunteers, "clinically unaffected" skin of patients with EB, and unaffected family members of EB patients (Table I, Fig 1). Three independent mutations to D-EBS-K are included. They span the quantitative interfamilial variation seen between different Köbner families. The EB22-patients are among the worst regarding persistent generalized blister liability (family XXII in [6]), while EB70-1 and EB87-1 had only moderate general blistering in infancy, with the later hand and foot blistering being more marked than seen in the local D-EBS-WC form of the disease. All developed blisters within the first week of life.

Fibroblast Cultures Cells from the established skin fibroblast cultures (some stored frozen) were subcultivated at passages 3–6 in disposable plastic culture dishes or flasks in 1 × Eagle's minimum essential (EME) medium (modified) with Earl's salt and 2.00 g/liter $NaHCO_3$ without glutamine, to which the following compounds were added (final concentrations in parentheses): fetal calf serum (15%), Hepes buffer pH 7.2–7.4 (5 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), nonessential amino acids (100 times dilution), and L-glutamine (2.00 mM) at 37°C in a 5% CO_2 atmosphere [10].

To determine gelatinase expression, each culture at a density of 4 × 10^3 cells/cm^2 was seeded in 3 T-75 flasks with serum-containing medium. At early confluence the cells were washed 3–4 times with Hanks' balanced salt solution and then 6 ml serum-free EME medium was added. After 24-h incubation the serum-free medium was havested. One part was stored intact and used to determine the amount of extracellular protein. The other part was made 0.2% (w/v) in BSA/50 mM in Tris-HCl (pH 7.5) and stored at −20°C before use in the gelatinase assay. The cell layers from these flasks were trypsinated, washed in saline, and the cell pellets obtained after centrifugation (600 *g* × 5 min) were stored at −20°C. These fibroblast pellets were diluted 1:4 with saline, sonicated, centrifuged at 14,000 *g* for 5 min at 4°C, and the supernatants obtained were used in the determination of lactic dehydrogenase (LDH) and esterase D (EsD) activities. Saline was added to 10-μl aliquots of these supernatants to give a final dilution of 1:99. This was used in the determination of the amount of total intracellular protein.

Gelatinolytic Activity Acid-soluble calf skin collagen was labeled with tritium by reductive methylation of the amino groups [11–13] to give labeled batches with specific radioactivities rang-

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Reprint requests to: Jan-Olof Winberg, Department of Genetics, The Norwegian Radiumhospital, 0310 Oslo 3, Norway.

Abbreviations:
BSA: bovine serum albumin
D-EBS-K: dominant epidermolysis bullosa simplex Köbner (generalized)
D-EBS-WC: dominant epidermolysis bullosa simplex Weber-Cockayne (localized)
EB: epidermolysis bullosa
EsD: esterase D
LDH: lactic dehydrogenase
SBTI: soybean trypsin inhibitor

Table I. Fibroblast Strains

Diagnosis/Code	Sex	Age (years)	Clinical/Electron Microscopy	Reference
D-EB Simplex Köbner				
EB22-1	F	65	Severe[a]	[6]
EB22-3	F	46	Severe	[6]
EB22-4	F	28	Severe	[6]
EB22-39	M	22	Severe	[6]
EB70-1	M	17	Mutant, moderate	[7]
EB87-1	M	3	Mutant, mild[b]	
D-EB Herpetiformis Dowling-Meara				
EB1-1	F	29	Classical[b]	[6]
Recessive junctional EB; R-EB Atrophicans Gravis Herlitz				
SEB4-1	F	8 mo	Classical	[8]
R-EB progressiva (late onset)				
EB57-100	F	19	Classical[b]	
Recessive dystrophic EB; R-EBD Hallopeau-Siemens				
EB75-1	F	11	Severe, mutilans	[9]
EB33-1	F	25	Mild[b]	[6,9]
R-EBD inversa				
EB11-1	F	30	Severe	[6,9]

Six healthy members of D-EB families and 3 other controls.
[a]Fig 1 in Appendix of [6].
[b]Electron microscopy by I. Anton-Lamprecht, Heidelberg, unpublished.

ing from 7 to 80 μCi/mg collagen. The gelatin substrate was prepared by first mixing 1 part of the tritium-labeled collagen (1 mg/ml and 30 μCi/mg) with 10 parts of unlabeled calf skin collagen (5 mg/ml 0.01 M acetic acid). Two parts of this collagen mixture (0.46% w/v) were added to 2.3 parts of 0.108 M Tris-HCl, pH 7.7, and heated at 60°C for 30 min giving a gelatin solution of 0.213% (w/v).

The gelatin-degrading enzyme(s) is/are exreted into the culture medium in a latent form and require(s) proteolytic activation. This was achieved as follows: aliquots of 0–100 μl of the crude culture medium were mixed with 0–100 μl of serum-free EME medium (contianing 0.2% μl to which 8 μl trypsin (2.5 mg/ml) was added. This mixture was incubated for 0–2 h at 35°C and the activation was terminated with 5 μl of SBTI (50 mg/ml). After 15-min incubation at room temperature, 50 μl of assay buffer (50 mM Tris-HCl pH 7.5, 0.852 M NaCl, 21.3 mM $CaCl_2$, and 0.852% BSA) and 50 μl of the 0.213% ^{3}H-labeled gelatin solution (1.065 nmol based on a molecular weight of 100,000/α-chain) were added. The gelatinase assay was carried out at 35°C for approximately 20 h. The reaction was terminated by adding 106 μl 45% trichloracetic acid to give final concentration of 15% [14]. After cooling on ice for 30 min, the assay tubes were centrifuged for 4 min using a Beckman Microfuge B. Then 160 μl of the supernatant was added to 5 ml Ready Solv HP and counted in a liquid scintillation spectrometer. The gelatinase activity obtained at optimal activation (Fig. 2*a*) always resulted in 10–20% degradation of the gelatin substrate. The assay is linear up to at least 30% degradation of the gelatine substrate as shown in Fig. 2*b*.

Other Assays The initial rate measurements to determine the activity of LDH and EsD were carried out on a recording filter fluorimeter, using an excitation wavelength of 328 nm and an emission wavelength of 460 nm. The LDH assay was carried out with fixed concentrations of 1 mM NAD and 1mM *d,l*-lactate in a total volume of 3 ml 0.1 M glycine-NaOH buffer pH 10.0 at 23.5°C [15,16]. The assay for EsD contained 0.1 mM 4-methylumbelliferyl acetate in a total volume of ml 10 mM sodium

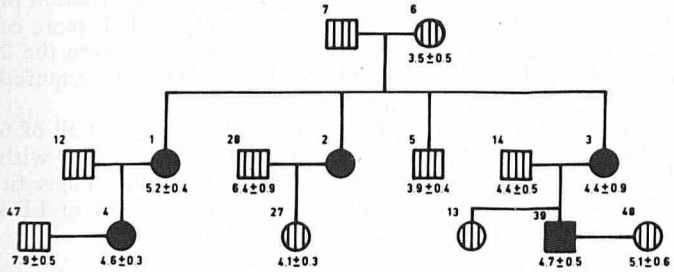

Figure 1. Pedigree of the D-EBS-K family EB22 (XXII in [6]). Gelatin degrading activity in nmol/h/mg protein (mean ± SD) is given under the symbols: unaffected (*striped box*)/affected (*solid box*) male; unaffected (*striped circle*)/affected (*solid circle*) female.

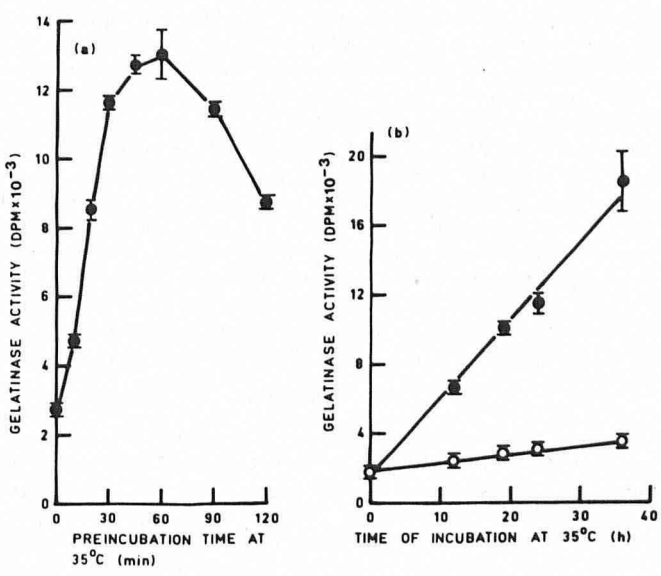

Figure 2. Gelatin degrading activity of crude culture medium from fibroblasts of a patient with D-EBS-K (EB22-1). Fifty μl of crude culture medium (1.49 μg protein) were proteolytically activated with 20 μg trypsin for either 0–2 h (*a*) or 1 h (*b*) at 35°C. All mixtures were assayed for gelatinase activity as described in *Materials and Methods*. The amount of gelatin degraded at maximum gelatinase activation was always calculated from 100% trypsin digestion of the gelatin substrate (66,000 ± 1,000 dpm). All assays were done at least in triplicate, with each point representing mean ± SD. *a*, Proteolytic activation curve. After preactivation all mixtures were assayed for 21.5 h at 35°C. *b*, Time dependence curve of gelatin degradation; *open circle*, unactivated crude culture medium; *solid circle*, proteolytically activated crude culture medium.

Table II. Gelatinolytic Protease, Lactic Dehydrogenase, and Esterase D Activities in Fibroblast Cultures

Culture	Number of Cultures	Gelatinase Activity			EsD Activity (nmol/min/mg protein[c]/cuvette)	LDH activity (nmol/min/mg protein[c]/cuvette)
		nmol/h/T-75	nmol/h/10^6 cell[a]	nmol/h/mg protein[b]		
Generalized D-EBS-K (N = 6)	8	0.82 ± 0.07	0.21 ± 0.02	4.5 ± 0.5	18 ± 4	253 ± 28
Controls (N = 3) (EBS relatives)	3	0.81 ± 0.12	0.22 ± 0.04	3.8 ± 0.3	17 ± 4	193 ± 50
Controls (N = 6) (non-EBS)	12	0.89 ± 0.06	0.20 ± 0.01	6.3 ± 0.6	18 ± 3	199 ± 27
Other EB (N = 6)	7	0.92 ± 0.09	0.28 ± 0.03	4.2 ± 0.6	22 ± 2	274 ± 20

[a]The number of cells per T-75 flask were counted on a Coulter Counter.
[b]Amount of extracellular protein.
[c]Amount of intracellular protein.

acetate buffer pH 5.5 at 23.5°C [17]. The reactions were started by the addition of 10 μl of sonicated cell solution. The amount of product produced was calculated from standard curves obtained with pure products (NADH and 4-methylumbelliferone).

To determine the amount of extra- and intracellular protein, the CBB G-250 dye method of Sedmak and Grossberg [18] was used. Standard curves were obtained with 0–2 μg BSA.

RESULTS

In this study of the expression of gelatinolytic activity in fibroblast cultures, optimal conditions as described by Sanchez et al [5] have been used. The integrity of the fibroblast cultures was tested by measuring the activity level of the 2 cytoplasmatic enzymes LDH and EsD. No significant differences between the cell cultures were found (Table II). Likewise, the amount of extracellular protein expressed in micrograms per T-75 flask varied within a range compatible with normal in vitro cell function. No systematic difference between the D-EBS-K patients (170 ± 20) and their unaffected relatives (209 ± 23), other EB patients (270 ± 46) or unrelated controls (153 ± 19) (mean ± SE) was found.

In Table II the gelatinase activity per hour is depicted in 3 different ways: per flask, per cell count, and per milligrams protein in the crude medium. Whereas all groups of subjects gave similar results using the first 2 ways, there was some variation when the activity was expressed per milligrams protein in the crude medium. No individual with grossly reduced gelatinase activity was found. The average of the cultures for the D-EBS-K patients is similar to the enzyme activity for other EB patients. They are insignificantly higher than for the unaffected relatives, yet somewhat below the values for the genetically unrelated controls. Fig 1 illustrates the individual variation within the EB22 family. Although on the whole below average for all the samples tested, reduced gelatinase activity is clearly demonstrated unassociated with the presence of the D-EBS-K gene in this family.

DISCUSSION

Dominant EB simplex can clinically and genetically be divided into at least 4 types: D-EBS-K (generalized), D-EBS-WC (localized), D-EBS Ogna (mild with fragile skin), and D-EBS mottled pigmentation (generalized) [1,19]. As these diseases are poorly characterized with respect to biochemical traits, genetic heterogeneity can be investigated if genetic linkage to a genetic marker system is demonstrated, i.e., a gene located beside the EB gene on the chromosome. A close genetic linkage to other genes enables us to distinguish between clinically and electron microscopically similar diseases. D-EBS Ogna has been distinguished as a separate genetic entity as it, in contrast to the other simplex forms, is closely linked to the GPT locus (on chromosome 8 or 16) [20,21]. However, D-EBS-K or D-EBS-WC genes have so far no confirmed assignment to a linkage group or specified chromosome.

The decrease in gelatinase activity in patients with D-EBS-K has been suggested to be a marker and hence a diagnostic tool for the Köbner form of the disease [5]. A similar defect in 6 of 13 D-EBS-WC patients indicated that some of the D-EBS-WC mutations could belong to the same gene locus as D-EBS-K [5]. In contrast to this, no decrease in gelatinase activity was found in any of our 6 D-EBS-K patients tested (representing 3 mutations) compared with controls. This excludes a defect in the gelatinase expression in fibroblast cultures being uniformly a marker for the D-EBS-K form of the disease. Since both studies used comparable, though slightly different methods for gelatinase expression, these results could be interpreted as genetic heterogeneity in the D-EBS-K group such as Sanchez et al [5] suggested for the D-EBS-WC group. However, in the absence of a parallel clinical heterogeneity, another possibility is that the gene locus for D-EBS could be immediately adjacent to the gene locus for gelatinase or a gene that regulates the gelatinase expression. Some mutations may therefore delete or involve expression of both loci and others only D-EBS. A prediction from this hypothesis is the existence of gelatinase mutations leaving the D-EBS locus intact.

It is important to stress that the gelatinase deficiency trait is not yet proven to be dominantly inherited, and its frequency in the general population is not well established. If present in EBS only by coincidence so that the materials are not selected with regard to gelatinase deficiency, the frequency in Norway should be less than 1/16 unrelated persons and in the United States population in the range 0/22 + 3/3 + 6/13? = 9/38. It would therefore be most profitable to study the United States families and population. We are currently proceeding with the study of Norwegian D-EBS-WC patients and families. From the unusually high mutation rate of 1.6/million gametes/generation of the pooled D-EBS-K and -WC genes [7], we acknowledge the possibility of more than one gene locus for these mutations.

The EBS–gelatinase puzzle is different from that related to the galactosylhydroxylysyl glucosyltransferase deficiency in the single Finnish family where 1 or 2 D-EBS patients were enzymologically normal and 2 non-EBS patients had the enzyme deficiency [4]. Rather than interpreting this as the effect of a single unusual mutant gene, which requires assumptions regarding nonpenetrance of a gene for generalized EBS [4], we consider loose genetic linkage the best interpretation vs chance cosegregation of 2 rare traits. The family gives maximum linkage (lod) score of +1.10 at a map distance of 18% recombination between the 2 disease loci, whereas +3 (i.e., odds for linkage 1000:1) is required to prove linkage.

The recent observation of Fine and Griffith [22] that all of 6 D-EBS-WC and 2 D-EBS-K patients had epidermal cells with an abnormal binding of peanut agglutinin, suggested a specific defect in glycosylation and therefore a new direction of EBS research.

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